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Bioanalytical method development and validation of atrasentan in human plasma using verapamil as internal standard by liquid chromatography coupled with tandem mass spectrometry

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***Abstract*---**A satisfactory LC-MS/MS separation and good peak symmetry for Atrasentan were obtained with Agilent, Zorbax, XDB C18 (2.1 x 50 mm ID, 5 µm), and a mobile phase containing a mixture of 5 mM Ammonium Formate buffer with 0.1% formic acid were mixed with HPLC grade Acetonitrile in the proportion of 70:30, v/v was delivered at a flow rate of 0.150 mL/min by positive ion mode (API 4000) with an injection volume of 10 µL and a run time of 3 min. Detection is performed by atmospheric pressure electrospray ionization (ESI) mass spectrometry in positive ion mode. The precursor to product ion transitions is *m/z* 5.11.600 to 354.04 for Atrasentan, and *m/z* 455.40 to 165.00 for Verapamil (Internal standard) were used for quantization. The retention time of Atrasentan and Verapamil (Internal standard) was found to be 1.68 & 0.96 min.

***Keywords*---**atrasentan, verapamil, LC-MS/MS, positive ion mode, internal standard.

Introduction

Atrasentan is a substance that is being studied in the treatment of cancer. It belongs to the family of drugs called endothelin-1 protein receptor antagonists. It is a novel, selective endothelin A receptor antagonist (SERA) (Dutta, S., Samara

et.al.). Atrasentan is chemically known as (2R,3R,4S)-4-(2H-1,3benzodioxol5yl)1[(dibutylcarbamoyl)methyl|2methoxyphenyl)pyrrolidine-3-carboxylic was shown in Figure 1. Verapamil (Internal Standard) is chemically known as 2-(3,4-dimethoxy phenyl)-5-{[2-(3,4dimethoxyphenyl)ethyl](methyl)amino}-2-(propane-2yl) pentane nitrile was shown in Figure 2. A literature review reveals that very few analytical methods have been reported for the determination of Atrasentan, which include UV-Spectrophotometry (Ahmad N et. al., 2021), High-performance liquid chromatography (Zhou Yet et., al, 2020, Heerspink HJL et.al 2018), HPTLC (Webb DJ et., al 2017) and LC-Mass spectroscopy method (Egido J et., al 2017). The present study aimed to develop a novel, simple, economical, and validated LC-MS/MS method for estimating Atrasentan according to FDA guidelines (Pena MJ et.al., 2017).

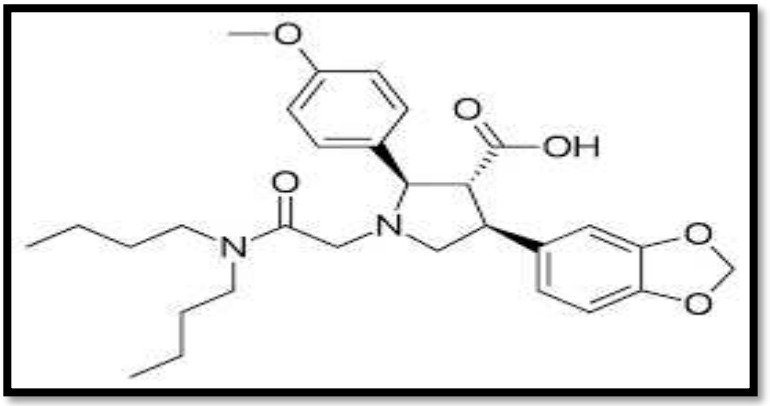


Figure 1: Chemical structure of Atrasentan

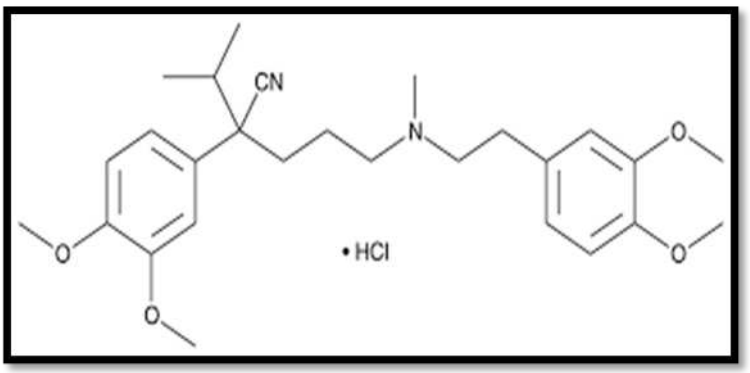


Figure 2: Chemical structure of Verapamil (Internal Standard)

Materials and Methods

Chemicals and Reagents

Atrasentan and verapamil (Internal Standard) were procured from Fisher chemicals, Mumbai, India. Acetonitrile of HPLC grade was procured from Rankem Ltd., India. The water of HPLC grade was obtained from Merck Specialties Private

Limited, Mumbai, India. Ammonium Acetate and formic acid of HPLC grade were procured from Merck Specialties Private Limited, Mumbai, India.

Instrumentation

An LC-MS/MS method was performed on a liquid chromatographic system consisting of a Waters Acquity UPLC system coupled with a Water Quattro Premier XE mass spectrometer with electrospray ionization (ESI) used for analysis, and Mass Lynx 4.1 SCN 805 software for processing and data collecting. Agilent, Zorbax, XDB C18 (2.1 x 50 mm ID, 5 µm) is used as a stationary phase.

Bio-analytical conditions

The chromatographic analysis was performed using a mobile phase of 5 mM Ammonium Formate buffer with 0.1% formic acid mixed with HPLC grade Acetonitrile in the proportion of 70:30, v/v with a flow rate of 0.150 mL/min by positive ion mode. Detection is performed by atmospheric pressure electrospray ionization (ESI) mass spectrometry in positive ion mode.

Mass spectrometry conditions

Acquisition duration: 3.0 min
Polarity: Positive
Scan Time: 200 milli seconds (for each MRM)
Resolution: Ql: Unit and Q3: Unit
Detection

	Q1 Mass	**Q3 Mass**
** Atrasentan	511.600	354.04
**** Verapamil	455.40	165.00

** Can vary by ± 0.5 Mass Units

Chromatographic Parameters

Equipment : Waters Acquity UPLC system coupled with a Water Quattro Premier XE mass spectrometer with electrospray ionization (ESI) and Mass Lynx 4.1 SCN 805 software
Column : Agilent, Zorbax , XDB C18 (2.1 x 50 mm ID, 5 µm)
Mobile Phase : 5 mM Ammonium Formate acetate buffer with 0.1% formic acid was mixed with HPLC grade Acetonitrile in the proportion of 70:30, v/v
Flow rate : 0.150 mL/min
Injection volume : 10 µL
Column oven temperature : 30±2ºC
Autosampler temperature : 10ºC
Run time : 3 minutes

Preparation of standard and working solutions for Atrasentan

The Atrasentan stock solution was prepared by dissolving 10 mg of Atrasentan 0.1% ammonium Formate solution in acetonitrile and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000µg/mL this solution was kept in the refrigerator at 2-8 °C. The stock solutions were diluted to suitable concentrations using diluent for spiking into Plasma to obtain calibration curve standards and quality control samples for further use. All other dilutions were made in the mobile phase.

Preparation of stock solution for Verapamil (Internal standard)

A stock solution of Verapamil (Internal standard) was prepared by dissolving 10 mg of verapamil in the mobile phase and making up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000µg/mL. This solution was kept in the refrigerator at 2-8 °C. Working IS solution was prepared by diluting the stock mentioned before use.

Preparation of calibration curve standards and quality control (QC) samples

A calibration curve standard consisting of eight non-zero concentrations ranging from 1 ng/mL to 1000 ng/mL of Atrasentan was prepared. Prepared quality control samples consisted of concentrations of 0.5 ng/mL (lower limit of quantification quality control sample), 3 ng/mL (lower quality control sample), 500 ng/mL (middle quality control sample), and 750 ng/mL (higher quality control sample) for Atrasentan These samples were stored at -70 °C ± 10 °C until use. In addition, twelve sets of LQC and HQC samples were stored at -20 °C ± 5 °C to check stability.

Preparation of plasma samples

For the preparation of plasma samples, human blood samples were collected into polypropylene tubes containing K_2-EDTA. Each tube was centrifuged for 15 min at 4500 rpm, and the supernatant was collected in another tube. To the supernatant, 1 mL of acetonitrile was added and kept for 10 min for the plasma proteins to precipitate, and then the supernatant was collected for further use.

Procedure for Spiked Human Plasma

Protein precipitation extraction was used to isolate Atrasentan and verapamil IS from Human Plasma. For this, aliquots of 20 µL of internal standard and 100 µL of plasma sample were added into labeled polypropylene tubes and vortexed briefly. Followed by the addition of 20 µL of diluent and vortexed. Then 20 µL of 0.1 % formic acid was added and vortexed. Followed by adding 5 mL of ammonium formate acetate and shaking for 30 min on a reciprocating shaker at 500 rpm. Next, samples were centrifuged at 2000 rpm for 10 min at 5 °C. Then supernatant organic layer (5.0 mL) was transferred to pre-labeled glass dry test tubes and evaporated to dryness in turboVap at 40 °C. Finally, the samples were reconstituted in 1000 µL of mobile phase containing 5 mM Ammonium formate

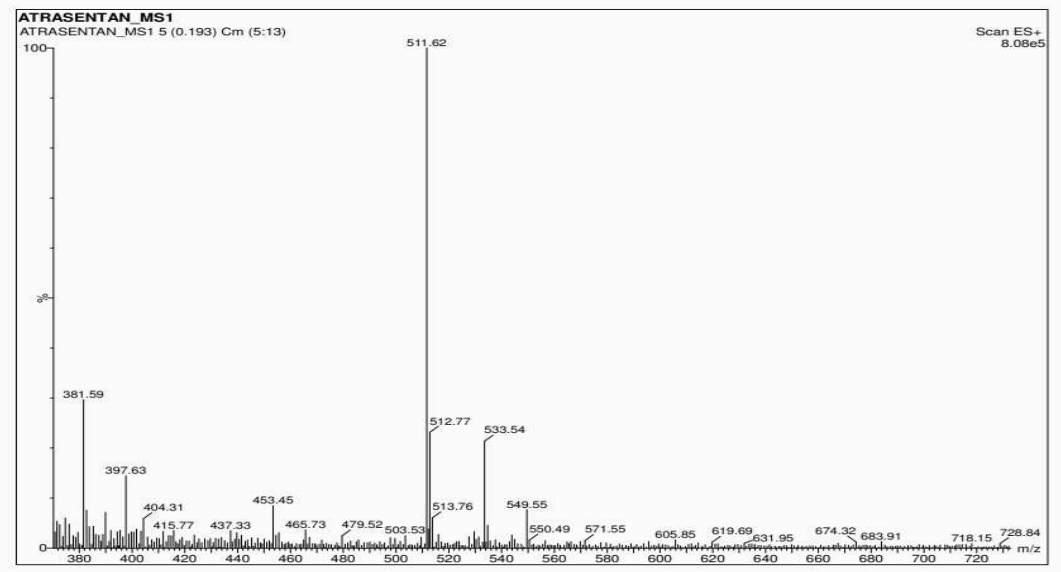
acetate buffer with 0.1% formic acid and mixed with HPLC grade Acetonitrile in 70:30, v/v, and 10 µL of the sample were injected to HPLC with MS-MS detection.

Preparation of sample solution

After bulk spiking, aliquots of 100 µL for calibration curves and 100 µL for quality controls of spiked plasma samples were pipetted into pre-labeled polypropylene microcentrifuge tubes. Then all the bulk spiked samples were stored in a deep freezer at -70 °C ± 10 °C, except twelve replicates each of LQC and HQC, which were stored at -20 °C ± 5 °C for generation of stability data. Finally, the thawed samples were vortexed to ensure complete mixing of the contents.

Optimization of LC/MS/MS method

For the optimization of the LC-MS/MS method, several parameters and mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Atrasentan were obtained with Agilent, Zorbax, XDB C18 (2.1 x 50 mm ID, 5 µm), and a mobile phase containing a mixture of 5 mM Ammonium formate acetate buffer with 0.1% formic acid were mixed with HPLC grade Acetonitrile in the proportion of 70:30, v/v was delivered at a flow rate of 0.150 mL/min by positive ion mode (API 4000) with an injection volume of 10 µL and a run time of 3 min. Detection is performed by atmospheric pressure electrospray ionization (ESI) mass spectrometry in positive ion mode. The precursor to product ion transitions is *m/z* 511.60 to 354.04 for Atrasentan, and *m/z* 455.40 to 165.00 for Verapamil (Internal standard) were used for quantization as shown in Figures 3 and 4. The retention time of Atrasentan and Verapamil (Internal standard) was found to be 1.68 & 0.96 min. A typical chromatogram of blank Plasma, Atrasentan, and Verapamil (Internal standard) is shown in Figure 5.



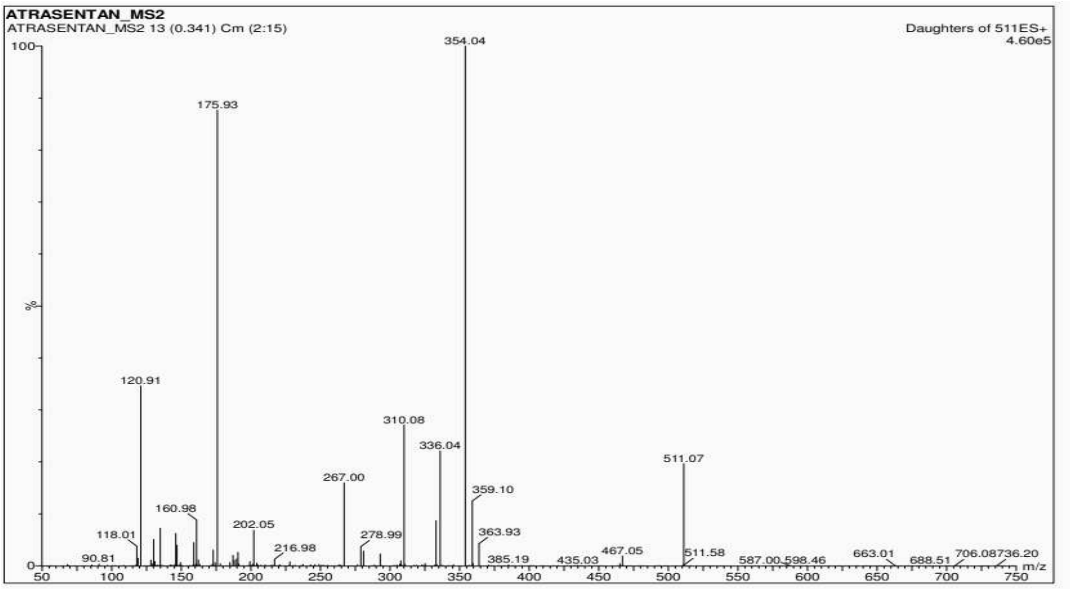
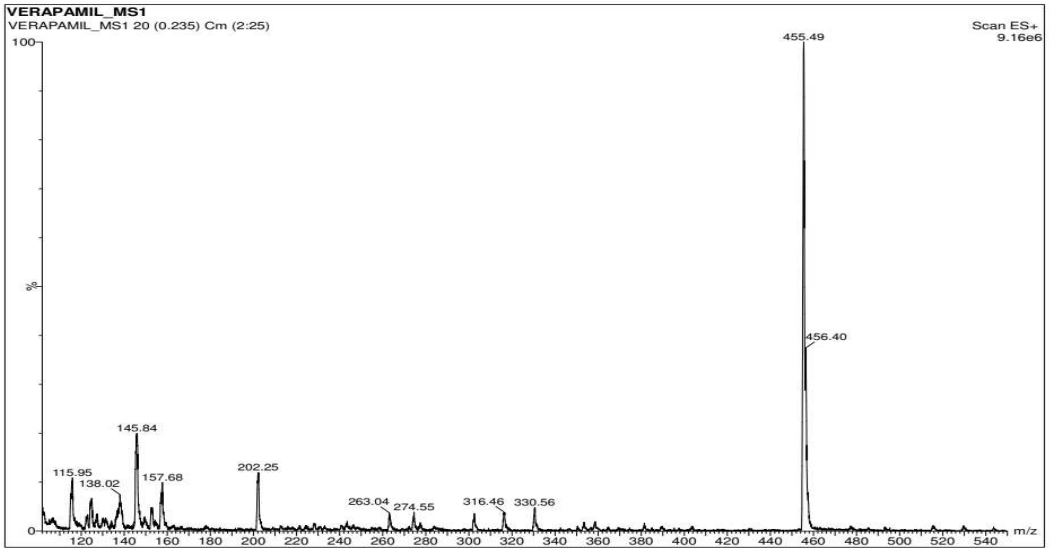


Fig. 3- Mass spectra of Atrasentan for precursor MS1 and product ion masses MS2



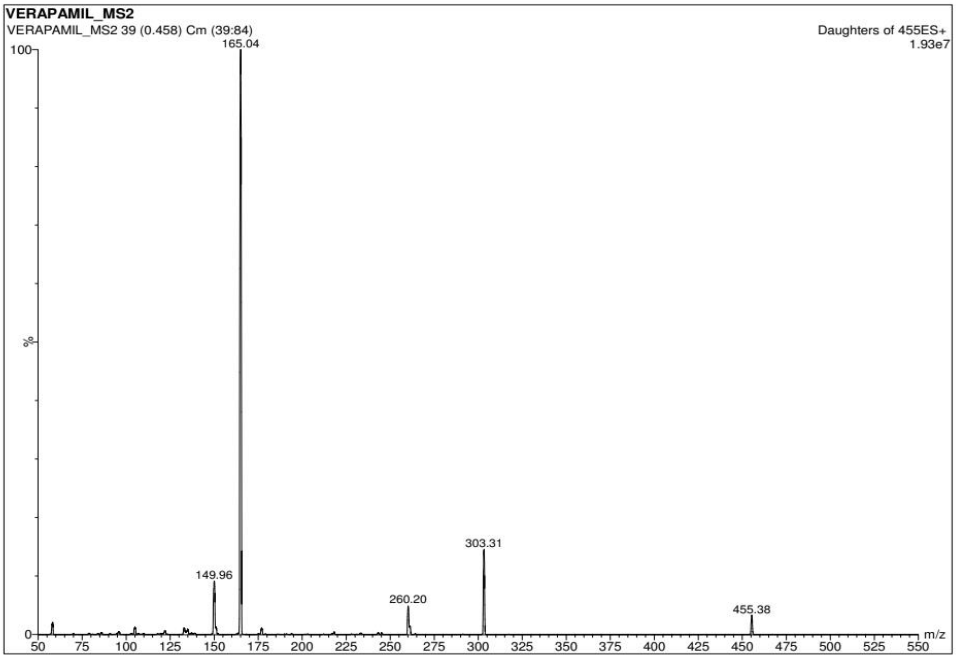


Fig. 4- Mass spectra of verapamil IS for precursor MS1 and product ion masses MS2

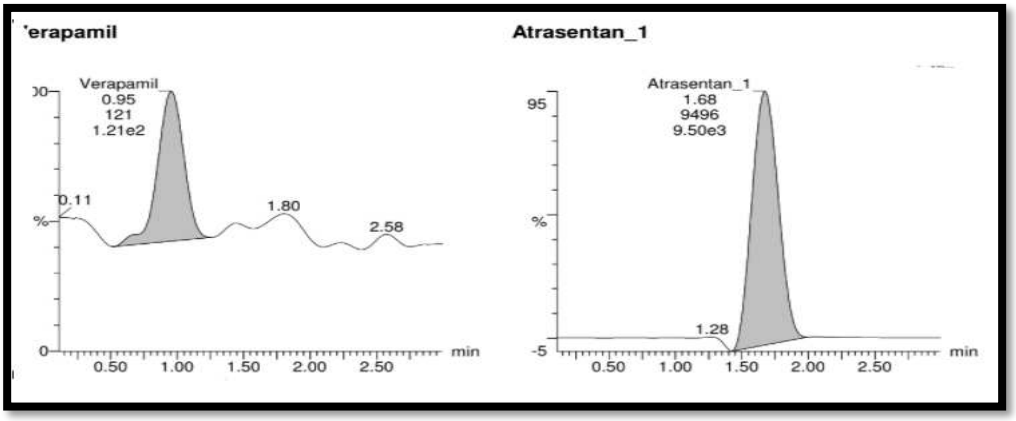


Fig. 5-Chromatogram of Atrasentan and verapamil (Internal standard)

Results and Discussion

A binary mixture of 5 mM Ammonium acetate buffer with 0.1% formic acid mixed with HPLC grade Acetonitrile in the proportion of 70:30 V/v was proved to be the most suitable mobile phase of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 0.150 mL/min Detection of the ions was performed by multiple reaction monitoring (MRM) of the transitions is *m/z 511.600* to 354.04 for Atrasentan and *m/z* 455.40 to 165.00 for Verapamil (Internal standard). The

retention time of Atrasentan and Verapamil (Internal standard) was found to be 1.68 & 0.96 min.

Linearity

The calibration curve was linear in the range of 1 ng/mL to 1000 ng/mL of the Atrasentan, as shown in Figure 6. A straight-line fit made through the data points by least square regression analysis showed a constant proportionality with minimal data scattering. The correlation coefficient (r) was 0.998 for Atrasentan, as shown in Table 1.

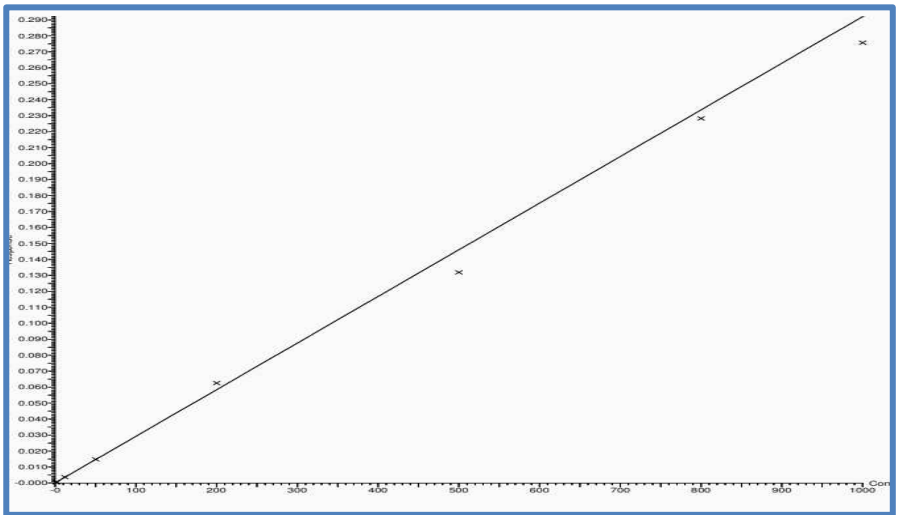


Fig. 6- Representative calibration curve for regression analysis of Atrasentan

Table 1: Linearity of Atrasentan

Concentration of Atrasentan (ng/mL)	Peak Area Ratio (Analyte area/IS area)
1	0.000
2	0.001
12	0.004
50	0.015
200	0.063
500	0.132
800	0.228
1000	0.276

Selectivity

No significant interference from endogenous components was observed at the mass transitions of Atrasentan and Verapamil (internal standard).

Recovery of the Atrasentan and Verapamil (Internal standard)

Recovery for Atrasentan was found to be in the range of 94.87% to 99.67%, and the mean recovery for Atrasentan was 96.24 %. While for verapamil (Internal standard), the mean recovery was 100.56 %.

Within-batch precision and accuracy

Within-batch precision for LLOQ quality control ranged from 2.12 % to 6.38 %, and for LQC, MQC, and HQC ranged from 0.9 % to 8.64%. Within-batch accuracy ranged for LLOQ quality control ranged from 99.5 % to 100.83 %, and for LQC, MQC, and HQC ranged from 97.01 % to 103.5 %.

Intra-day precision and accuracy

Intra-day precision for LLOQ quality control was 4.173 %, and for LQC, MQC, and HQC ranged from 1.486 % to 6.779%. Intra-day accuracy for LLOQ quality control was 102.42 %, and LQC, MQC, and HQC ranged from 98.31 % to 102.46 %.

Between batch/inter-day precision and accuracy

Between batch precision for LLOQ quality control was 4.886 % and for LQC, MQC, and HQC ranged from 1.558 % to 6.753 %. Between batch accuracy for LLOQ quality control was 100.56 % and for LQC, MQC, and HQC ranged from 98.18 % to 102.21 %.

Stability

The processing and storage conditions of clinical samples need to maintain the integrity of a drug or at least keep the variation of pre-analysis as minimal as possible. For this reason, stability studies play an essential role in bioanalytical method development. This study assessed the stability by considering different studies such as room temperature, solution stability, refrigerated stock solution stability, and bench top stability. The results show that Atrasentan is stable under the studied conditions since the international acceptance criteria (variation values for areas smaller than 15 %) were met in all cases.

Room temperature stock solution stability

The stability was found to be 99.39 % for Atrasentan, with the precision ranging from 9.5% to 12.54%. The stability was found to be 101.06 % for verapamil (Internal standard), with the precision ranging from 3.43 % to 5.69 %. The results of room temperature stock solution stability are shown in Table 7.

Refrigerated stock solution stability (at 2-8 °C)

The stock solution was found to be stable for four days. The four days' stock solution stability of Atrasentan and verapamil (Internal standard) was found to be 93.21 % and 101.3 %, respectively, as shown in Table 8.

Bench top stability

Atrasentan was stable for up to 6 hours as per the acceptance criteria. The percent mean nominal ranged from 98.9 % to 101.4 %, and the precision ranged from 2.68 % to 4.9 %. Results of bench top stability are shown in Table 9.

Table 7: Room temperature stock solution stability of Atrasentan and verapamil (Internal standard) for 0 and 6 hours

S. No.	Atrasentan Peak Area		verapamil (Internal standard) Peak Area	
	0 hr	6 hr	0 hr	6 hr
1	32270	30198	16887	16145
2	29668	29938	16224	15789
3	34706	32876	15458	14863
4	30284	31284	14789	16376
5	27099	27899	15925	15500
6	24170	24870	14534	15490
Mean	29699.50	29510.83	15636.17	15693.83
SD	3724.33	2802.09	889.69	538.03
CV %	12.54	9.50	5.69	3.43
Stability %	99.39		101.06	

Table 8: Refrigerated stock solution stability of Atrasentan and verapamil (Internal standard) at 2-8 °C for 4 days

S. No.	Atrasentan		verapamil (Internal standard)	
	Stability standard stock	Comparison standard stock	Stability standard stock	Comparison standard stock
	Peak Area	Peak Area	Peak Area	Peak Area
1	30803	30343	15145	15490
2	30420	30850	15789	15358
3	30011	30641	14863	15755
4	30587	30457	15376	15191
5	30431	29988	15500	15713
6	30069	31269	15490	13810
Mean	30386.83	30591.33	15360.50	15219.50
SD	302.85	440.98	320.63	722.52
CV %	1.00	1.44	2.09	4.75
N	6	6	6	6
Mean response of standard stock	30489.08		15290.00	
Mean standard response	32711.00		15000	
Response %	93.21		101.3	

Table 9: Benchtop stability of Atrasentan for 6 hours

S. No.	Concentration (ng/mL)	
	LQC	HQC
	2	780
1	2.03	795.9
2	1.89	770.5
3	2.11	781.5
4	1.95	749.8
5	2.2	743.8
6	2.03	785.9
Mean	2.03	771.23
SD	0.10	20.70
CV %	4.90	2.68
Nominal %	101.4	98.9
N	6	6

S. No.	Concentration (ng/ml)	
	LQC	HQC
	2	780
1	2.1	770.5
2	2.01	781.5
3	2.03	749.8
4	2.01	781.5
5	2.03	749.8
6	1.89	765.9
Mean	2.01	766.50
SD	0.07	14.31
CV %	3.39	1.87
Nominal %	100.6	98.3
N	6	6

Matrix effect

No significant matrix effect was observed in all the eight batches, including hemolysis and lipemic Plasma for Atrasentan at low (LQC) and high (HQC) concentrations. The precision and accuracy for Atrasentan at LQC concentration were found to be 1.43 % and 102.3 %, respectively, and at HQC concentration was found to be 2.59 % and 97.1 %, respectively, as shown in Table 10.

Table 10: Matrix effect of Atrasentan

Plasma (Batch No.)	LQC (2 ng/mL)			Mean	HQC (780 ng/mL)			Mean
	1	2	3		1	2	3	
1	2.2	2.03	2.01	2.07	749.8	770.5	768.1	770.50
2	1.97	2.01	2.03	2.00	765.9	781.5	749.8	781.50
3	2.3	2.03	1.89	2.07	792.5	749.8	765.9	749.80
4	2.01	1.95	2.11	2.02	795.9	743.8	792.5	743.80

5	2.03	2.2	1.95	2.05	770.5	785.9	787.9	785.90
6	2.01	2.04	2.2	2.07	734.9	756.1	772.8	756.10
(Lipemic)	1.95	2.2	2.1	2.07	781.5	738.1	781.5	738.10
(Hemolytic)	2.05	1.97	2.01	2.01	764.9	734.9	781.5	734.90
Mean				2.05	Mean			757.58
SD				0.03	SD			19.59
CV %				1.43	CV %			2.59
Nominal %				102.3	Nominal %			97.1
N				8	N			8

Conclusion

The present LC-MS/MS method for estimating Atrasentan in human Plasma by using verapamil as an internal standard was established and validated as per FDA guidelines. A simple and inexpensive liquid-liquid extraction procedure and an isocratic chromatography condition using a reversed-phase column provided an assay well suited for real-time analysis. This method was intended to rapidly and accurately estimate Atrasentan in Human Plasma. The chromatographic peaks were separated, and no interfering peaks were found. Several commercially available HPLC columns and various mobile phases were used to develop the LC-MS method for estimating Atrasentan in Human Plasma. A satisfactory LC-MS separation and good peak symmetry for Atrasentan were obtained with Agilent, Zorbax, XDB C18 (2.1 x 50 mm ID, 5 µm), and a mobile phase containing a mixture of 5 mM Ammonium Formate buffer with 0.1% formic acid were mixed with HPLC grade Acetonitrile in the proportion of 70:30, v/v was delivered at a flow rate of 0.150 mL/min by positive ion mode (API 4000) with an injection volume of 10 µL and a run time of 3 min. Detection is performed by atmospheric pressure electrospray ionization (ESI) mass spectrometry in positive ion mode. The precursor to product ion transitions is m/z 5.11.600 to 354.04 for Atrasentan, and m/z 455.40 to 165.00 for Verapamil (Internal standard) were used for quantization. The retention time of Atrasentan and Verapamil (Internal standard) was found to be 1.68 & 0.96 min. Linearity was established for Atrasentan in the range of 1 ng/mL to 1000 ng/mL with a correlation coefficient (r=0.998), and the overall percentage recovery was 96.24 % for Atrasentan and 100.56 % for verapamil (Internal standard) respectively. The CV % values of accuracy and precision for Atrasentan were found to be ≤ 15 %, indicating the proposed method's accuracy and precision. The CV % values of accuracy and precision of Atrasentan for stability studies were ≤ 15 %, indicating the stability of the proposed method. The LC-MS/MS method for estimating Atrasentan in human Plasma using verapamil as an internal standard exhibited excellent performance in selectivity, linearity, accuracy, precision, recovery, stability, and matrix effect test. In addition, the reported method has a short analysis run time, an advantage over previously reported methods. Therefore, this method is suitable for therapeutic drug monitoring of Atrasentan.

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